

# PURIFICATION AND CHARACTERIZATION OF LIPASE ENZYME FROM PHASEOLUS VULGARIS (FRENCH BEAN) SEEDS AND SESAMUM INDICUM (SESAME SEEDS) AND THEIR SYNERGISTIC ACTION IN MEDICAMENTS FOR ATHEROSCLEROSIS

**HARIHARAN P<sup>\*1</sup>, LALITHA KAVYA KOSURU<sup>2</sup> & NAGENDRA H G<sup>1</sup>**

<sup>1</sup>Downstream Bioprocessing Laboratory, Department of Biotechnology, Sir M Visveavaraya Institute of Technology,  
Yelahanka, International Airport Road, Bangalore 562157, Karnataka, India

<sup>2</sup>Masters in Biomedical Technologies, Faculty in Medicine, Eberhard Karls University of Tubingen, Germany

## ABSTRACT

*This study is taken for the screening of lipolytic enzymes from plant seeds to degrade the lipids by regulating several signaling proteins in the progression of atherosclerosis. Bioactive components from Phaseolus vulgaris (French bean) seeds and Sesamum indicum (sesame seeds) have been studied for their degradation properties of triglycerides and cholesterol by titration and plate assay method. The enzyme was purified to homogeneity by Ammonium sulphate fractionation at 60% saturation, with an overall yield of 26% and 1.3 fold purification. Molecular mass of is ~30 kDa. The ideal pH required for the enzyme activity was observed to be 7.0 and was stable at a pH extend 6.0 to 8.0, optimum temperature was 40°C and 100% stable even incubated for 2 h. The metal ions Ca<sup>2+</sup>, Mg<sup>2+</sup>, improved relative activity of the enzyme while EDTA and Hg<sup>2+</sup> were hindered the enzyme activity. The molecular weight of the lipolytic proteins from Sesamum indicum and Phaseolus vulgaris showed above 30kDa. According to literature review plants based enzymes molecular weight range from 12kDa to 27kDa. Although the enzyme from plant source showed high molecular weight comparing to its usual range. Hence this lipolytic enzyme has diverse applications in pharmaceutical industry for is substrate specificity, enantioselective and with higher molecular weight.*

**KEYWORDS:** Phaseolus vulgaris (French bean) seeds and Sesamum indicum (sesame seeds), Lipase enzyme, Purification, atherosclerosis, etc.

**Received:** Feb 17, 2022; **Accepted:** Mar 02, 2022; **Published:** Mar 11, 2022; **Paper Id.:** IJBTRJUN202201

## INTRODUCTION

Atherosclerosis is a cardiovascular disease which occurs due to the plaque accumulation in the intima region of arterial walls. Plaque is formed as a result of LDL taken up by macrophages to form foam cells. Plaque build-up in the intima where VSMCs are present will proliferate the lipid within the cells. Plaques may rupture, causing acute occlusion of the arteries, which leads to High blood pressure, Chest pains and Heart attacks. In severe conditions, the plaque needs to be extracted by surgical methods, though most patients would prefer non-surgical interventions. The surgical interventions and diagnostics like angioplasty and angiogram will cost 1.5 to 3 lakhs and the medications will easily cost estimation per year is 2 lakhs. To reduce the cost of the medications the lipolytic proteins from plant seeds are isolated and characterized of their lipid lysis activity.

Lipolytic enzymes has a critical role in reducing lipid degradation in the form of fatty acids by breaking the glycerol bonds in triglyceride. Lipases can be extracted from microbes, animals and plant seeds. Literature studies indicate that seed based lipases are more selective towards the target. Stereochemical evidence suggests

that plant based therapeutic enzymes are highly selective towards the target and also enantioselective.

This study is taken for the screening of lipolytic enzymes from various plant seeds to degrade the lipids by regulating several signaling proteins in the progression of atherosclerosis. Bioactive components from *Phaseolus vulgaris* (French bean) seeds and *Sesamum indicum* (sesame seeds) have been studied for their degradation properties of triglycerides and cholesterol by titration and plate assay method. The molecular weight of the lipolytic proteins from *Sesamum indicum* and *Phaseolus vulgaris* showed above 30kDa. According to literature review plants based enzymes molecular weight range from 12kDa to 27kDa. Although the enzyme from plant source showed high molecular weight comparing to its usual range. Hence this lipolytic enzyme has diverse applications in pharmaceutical industry for its substrate specificity, enantioselective and with higher molecular weight.

## Materials and Methods

Seeds of *Sesamum indicum*, and *Phaseolus vulgaris* procured from agricultural markets and grocery stores. Olive oil purchased from the grocery store, Agar-Agar, Tween 20, Methylene red dye, HCL (0.1N), NaOH 0.1N), Sodium desoxycholate, pH buffers, Phenolphthalein indicator, Distilled water, Mortar and Pestle, Homogenizer, Centrifuge, Laminar air flow, pH meter, Burette and Micro Pipettes.

## Collection of Samples

Samples were collected from agricultural seed market.

## Germination of the Seeds

Seeds are washed 2-3 times to eliminate any dirt and to reduce fungal contamination. In a petri dish the sterilised tissue paper is laid and the washed seeds are placed on the tissue paper followed by the closing the lid of petri dish.

Small amount of water was added to tissue paper to not let the seeds dry out. This process was repeated 2-3 days until seeds were sprouted.

## Enzyme Extraction

Sprouted seeds were homogenized in mortar and pestle with minimum water till their physical structure change to a slurry. This slurry is centrifuged at 10,000rpm for 10 mins, supernatant after centrifugation is crude enzyme.

## Purification of Crude Enzyme

- Ammonium salt fractionation –The common step to purify crude enzyme is by salt fractionization at high osmotic mediums like salt solutions. Protein in crude form are fractionized in step by step increase the amount of salt or its ionic strength is an effective strategy for obtaining partially purified enzymes.
- Dialysis- Dialysis is the secondary step of purification process after salt fractionization, it separates the desired protein from other unwanted molecules such as salt in the presence of semipermeable membrane. This membrane contains micropores through which small molecules in protein sample will pass out and come out in the dialysate, this moving of molecules through the semipermeable membrane is known as diffusion.
- Ion exchange chromatography - Ion exchange chromatography is used to separate any charged molecule, such as bulky proteins, amino acids, and nucleotides. This separation method is also used for analytical and preparative

purposes. DEAE ion exchanger is an anionic exchanger which traps negatively charged particles and release the low density positively charged particles.

## ENZYME ASSAY

### Substrate Preparation:

2gms olive oil is weighed in a beaker. Oil is emulsified with the bile salts. Sodium deoxycholate is also a bile salt. Sodium deoxycholate is weighed 100mg and dissolved in 25ml of distilled water, 20ml of bile salt solution is added to the olive oil till it gets emulsified to a milky white colour.

## TITRIMETRIC METHOD

- Reaction mixture consists of 1ml of crude enzyme, 5ml of substrate and 2ml of phosphate buffer (50mM).
- Set the pH at 7.00
- Keep the reaction mixture for 30 min incubation with shaking at 37° Celsius.
- Reaction was stopped by adding 4ml of acetone: ethanol (1:1) containing 0.09% phenolphthalein indicator.
- Enzyme activity is determined by titration of fatty acid released by 50mM NaoH.

$$\text{Enzyme activity} = \frac{\text{volume of alkali consumed} \times \text{strength of alkali}}{\text{Wt of sample in grams} \times \text{time in min.}}$$

## PLATE ASSAY METHOD

The principle to measure the lipase activity is the micromoles of fatty acid released from the substrate. According to (Mohd yusuf abd samad 1989) the plate assay for lipase activity measurement was the zone of clearance around the well where enzyme is loaded.

- Plates are prepared by adding 2.5% agar, Methyl red as indicator and 2% substrate.
- Wells are made the help of well borer.
- Initial concentration is 10µl and other wells are 1:1 and 1:2 dilutions of the enzyme. Diluent used here is distilled water.
- These plates are kept for incubation for 24 to 72hrs.

## CHARACTERIZATION OF THE LIPOLYTIC ENZYME

### Effect of Temperature:

The optimum temperature for maximal lipase enzyme activity was determined by incubating 100µl of enzyme with 0.9ml pH7.0 phosphate buffer at different temperatures ranging from 20°C to 60°C using Olive oil emulsion as a substrate. The enzyme's thermal stability was observed in the 20°C-60°C optimal temperature range. The enzyme activity was measured after it was incubated for varied time intervals (40, 80, 120, and 160 minutes).

### Effect of pH:

Effect of pH on purified enzyme is studied by addition of 100µl of sample enzyme to 0.9ml of various buffers from pH ranging from (1.0 to 10) for 15 mins at room temperature. Olive oil emulsion of 1ml is added to the enzyme in various

buffers and distilled water is added to make the volume to 2ml and incubated in room temperature for 10 mins and enzyme activity was measured. Buffers used in the experiment were Phosphate buffer (pH 5.0 and 10) and Tris HCL (1.0 -4.5).

#### Effect of Metal Ions:

The effect of metal ions on purified enzyme was checked by incubating 100µl of enzyme sample with 0.25ml of 1mM conc. of solutions of various metals ions like Hgcl<sub>2</sub>, Cacl<sub>2</sub>, Mncl<sub>2</sub>, Mgcl<sub>2</sub>, Zncl<sub>2</sub> and EDTA and 0.65ml of phosphate buffer of pH7 at room temperature for 20mins. After incubation oil emulsion is added to reaction mixture and enzyme activity is measured and control activity of sample was measured without the addition of metal ions.

#### SDS PAGE Molecular Weight Determination:

To determine the molecular weight and purity of the purified enzyme was carried through SDS polyacrylamide gel electrophoresis using 6% stacking gel and 12%separting gel. The sample loading buffer consists of Tris HCL pH 6.8, 5ml, SDS : 0.5g sucrose, bromophenol blue. The partially purified sample is added to sample loading buffer. This sample was loaded into wells with sample loading buffer and the subjected to electrophoresis with a constant voltage supply of 50mv. After the electrophoresis, the gel is stained with 0.02% of Coomassie blue which is dissolved in methanol and acetic acid 6-12 hours.

The stained gel is de-stained by de-staining solution consists of methanol and acetic acid and post de-staining photograph is taken. The molecular weight of the sample was determined by comparing the standard marker proteins.

## RESULTS AND DISCUSSIONS

#### Enzyme Activity by Titration

Titration of milky textured emulsion (white in colour), after bio catalysis the white colour will turn into pale pink as its pH is increased.

NO. of Trials	<i>Sesamum Indicum</i> (U)	<i>Phaseolus vulgaris</i> (U)
1.	1200	1220
2.	1170	1125
3.	1250	1182
4.	1198	974
5.	1235	1046
Average enzyme activity	1211	1108

#### Enzyme Activity by Plate Assay Method:

Substrate used in this assay is both olive oil and tween 20. Substrate preparation: To prepare the substrate Olive oil is treated with sodium deoxycholate to make white creamy emulsion, substrate 2% of the substrate is added to the agar and inclusion of dye will make the zone clear.

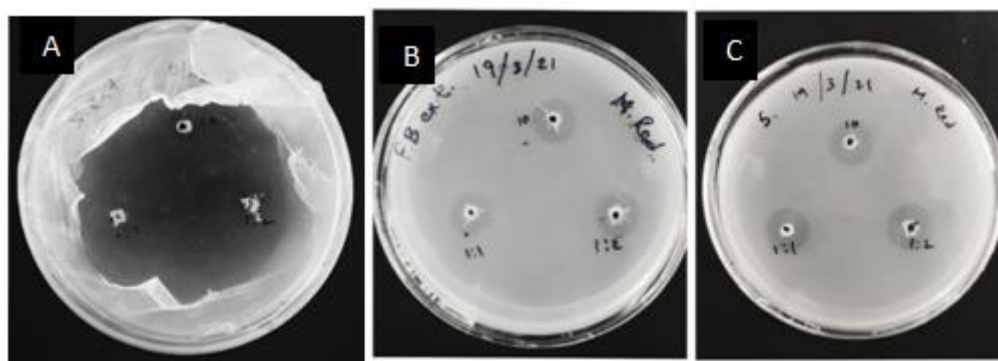


Figure 1: (A) Control, (B) Zone of Clearance is from *Phaseolus Vulgaris* and (C) *Sesamum Indicum* and Substrate as Olive Oil Emulsion

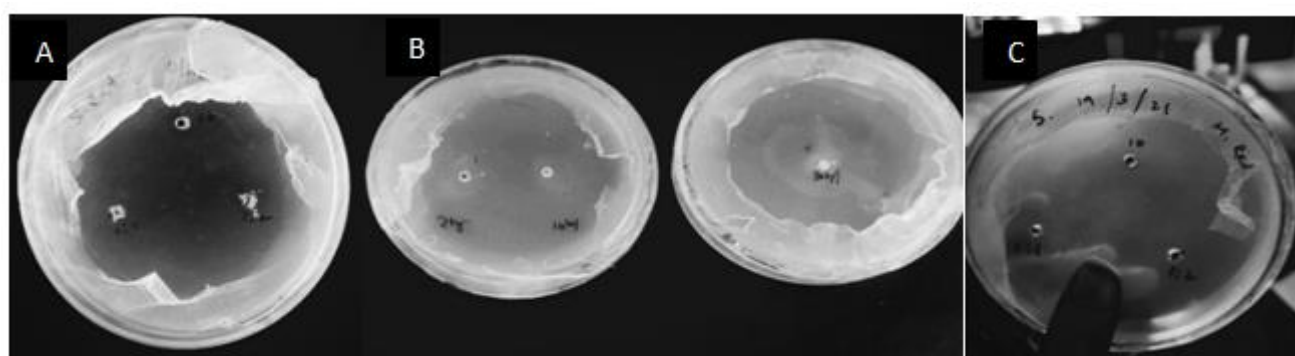


Figure 2: (A) Control (B) *Phaseolus Vulgaris* (Middle) Showing Zone of Hydrolysis and (C) *Sesamum Indicum* (Right) Lipase is against Tween-20 as Substrate

Samples	Substrate	Types of Zones	Incubation Time
Sesamum indicum, <i>Phaseolus vulgaris</i>	Oil	Equally distributed circular zones	48-72 hrs
<i>Phaseolus vulgaris</i>	Tween 20	Irregular zones	48-96 hrs

## Observations

Table 1

Samples	Olive oil emulsion	Tween 20
Sesamum indicum	+	—
<i>Phaseolus vulgaris</i>	+	+

The overall Plate assay analysis shows that *Sesamum indicum* shows lipolytic activity with a specific substrate of olive oil. Furthermore, *Phaseolus vulgaris* shows the lipase activity on both the substrate (olive oil and tween-20). Although *Phaseolus vulgaris* do not show the circular and defined zones on Tween-20.

## Purification

The extraction and purification of lipolytic enzyme at optimized conditions resulted in gradual increase in specific activity

at every stage of purification. The lipolytic enzymes from *Sesamum indicum* and *Phaseolus vulgaris* were purified up to 3.5 and 3 fold as compared to crude enzyme.

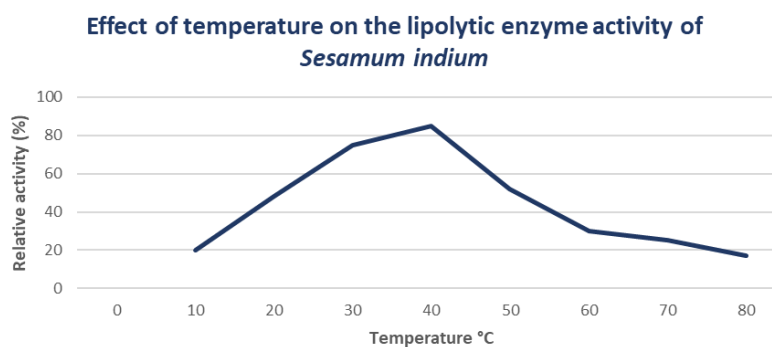
**Table: 3 Lipase Enzyme from *Sesamum Indicum***

Process	Protein (mg)	Activity	Specific Activity (U/mg)	Yield (%)	Fold Purification
Crude enzyme	92	1250	13.6	100	1
Salt fractionization	68	1100	16.2	88	1.1
Dialysis	46	1035	22.5	83	1.6
DEAE-Cellulose	18	856	47.5	68	3.5

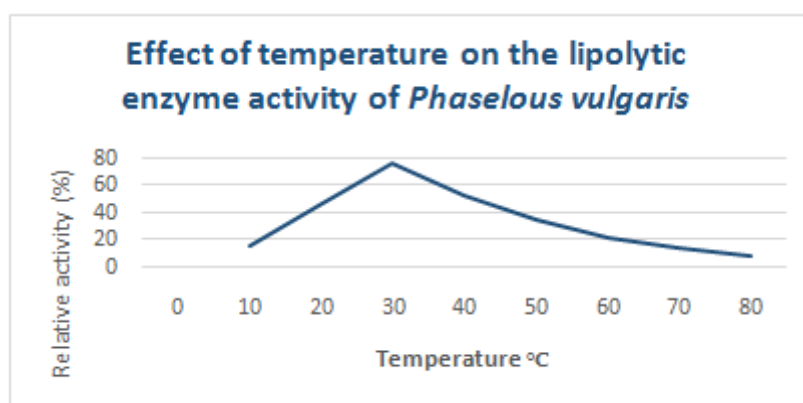
**Table 4: Lipase Enzyme from *Phaseolus vulgaris***

Process	Protein (mg)	Activity	Specific Activity (U/mg)	Yield (%)	Fold Purification
Crude enzyme	98	1220	12.4	100	1
Salt fractionization	56	1138	20.3	93	1.6
Dialysis	49	1105	22.5	90	1.8
DEAE-Cellulose	25	974	38	80	3

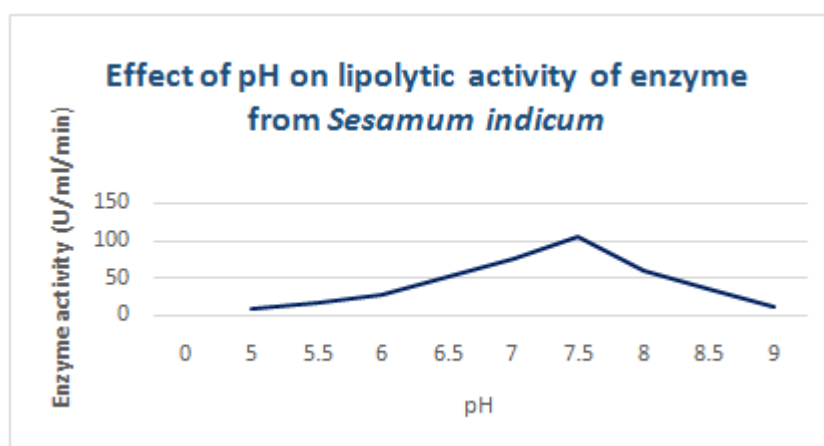
## CHARACTERIZATION OF LIPASE



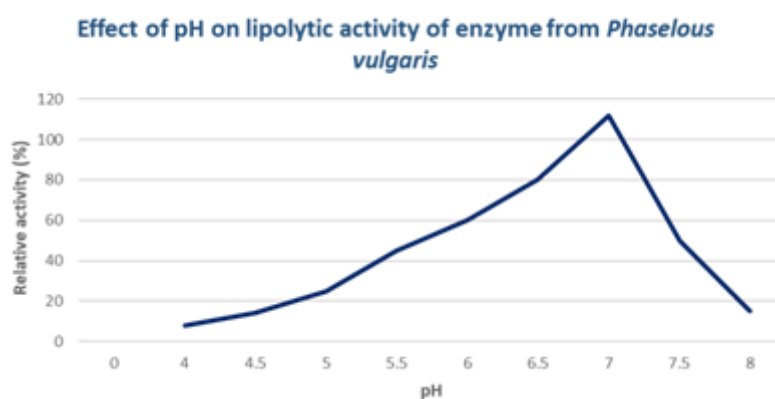
The Optimum temperature of *Sesamum indicum* is 38°C. However as the temperature increases the enzyme activity decreases gradually after 40°C. Lipase enzyme showed minimal activity at 10°C and above 50°C.



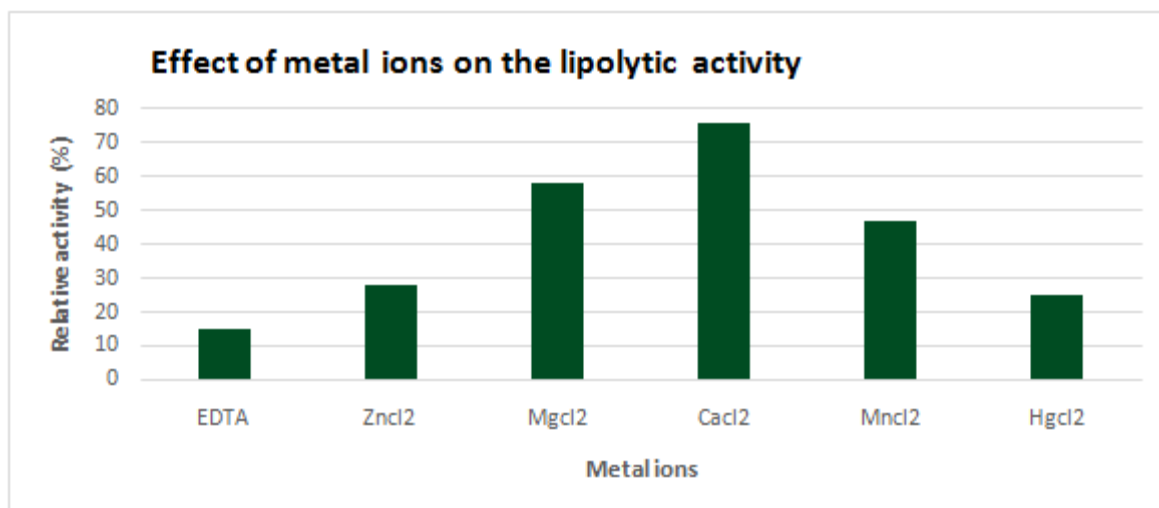
The Optimum temperature of *Phaseolus vulgaris* is 35°C. However temperature increases the enzyme activity decreases gradually before crossing 40°C. Lipase enzyme showed minimal activity at 20°C and above 50°C.



The enzyme from *Sesamum* showed maximum activity which can be concluded that the optimum pH for *sesamum* lipase is 7.5 and pH drastically dropped at 8 and above.



The enzyme showed maximum activity which can be concluded that the optimum pH for *Phaseolus* is pH 7 and the pH drastically dropped at 8 and above.



To determine the stability of lipase enzyme in the presence of metal ions, the effect of metal ions on purified lipase enzyme is performed.

Calcium chloride interaction with lipase increased the lipolytic activity of the enzyme, and magnesium chloride showed the second best reaction for lipase activity. Lipase in the presence of EDTA showed the minimal activity.

#### SDS PAGE:

The purified lipolytic enzyme along with standard molecular markers was run on the SDS PAGE, the standard molecular weight markers are bovine serum albumin (66kDa), ovalbumin (45.0kDa), lactate dehydrogenase (31.0kDa) lactoglobulin (14.0 kDa) and rease bsp981 (21.5kDa). According to some researchers the molecular weight of intra cellular lipase 65K Da (Jayati ray et al., 2015)

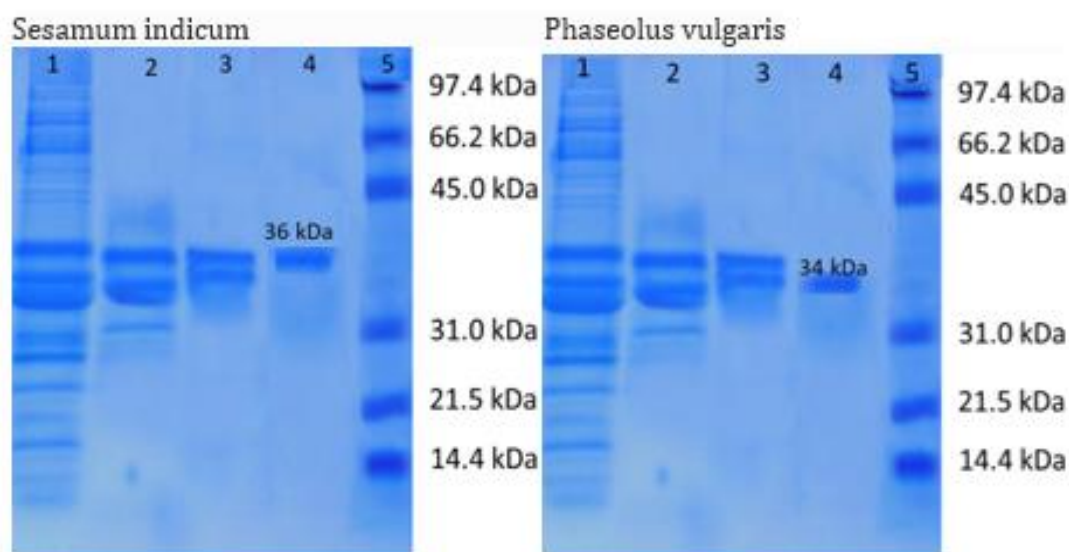


Fig4.3.34 1). Crude enzyme, 2). Ammonium sulphate precipitation, 3) Dialysis, 4) DEAE-Cellulose ion exchange chromatography 5)Molecular biomarkers

The determination of molecular weight of SDS PAGE shows that *Sesamum indicum* and *Phaseolus vulgaris* approximately 36 kDa and 34 kDa respectively.

## CONCLUSIONS

The present research was conducted to screen the lipolytic proteins from plant seeds in their germination stage. These enzymes are identified to show the lipolysis activity on various substrates such as Tween-20 and Olive oil. The lipolytic activity was measured by various assays namely titration and Plate assay analysis which shows lipolytic activity in the form of zone of hydrolysis. Plant seeds especially oilseeds at germinating stage shows the higher lipolytic activity and have the potential of risen ability of their pharmaceutical applications. The signalling proteins involved in the progression of atherosclerosis can be controlled by bioactive components like sesamin, sesaminol, saponins, propionate, Phyto-hemagglutinin and sterols from *Sesamum indicum* and *Phaseolus vulgaris*. Atherosclerosis being a CVD, takes time for its progression, the plaque build-up, clotting of blood and high cholesterol will eventually lead to the atherosclerotic lesion.

As per the biochemical characterization and stability studies results showed that lipase enzyme is stable at pH 7 and body temperature unlike other lipolytic enzymes which are stable at below pH 4 also. Lipase stability at pH 7 gives possibilities to for many pharmaceutical application as it will reduce the expense of artificial environment for its stable activity. Plant seed based lipolytic enzymes are highly selective to the target and this research can support the other lipolytic proteins which have not been explored in the plants.

The plant based lipases generally have molecular weight of 12kda to 27 KDa (Vaijanti Mala, 2004, A Review of Enzymatic Properties of Lipase in Plants, Animals and Microorganisms) Although biochemical characterization studies showed that our enzyme is having the molecular weight of 34k Da and 36 kDa of *Phaseolus vulgaris* and *Sesamum indicum* respectively, which gives the novelty of high molecular weight of proteins from plant source. The enzyme from *Phaseolus vulgaris* and *Sesamum indicum* showed high stability at neutral pH and body temperature, also plant based proteins are highly enantioselective which enables that lipolytic proteins from *Phaseolus vulgaris* and *Sesamum indicum* can be applied in atherosclerosis medication with animal studies.

## ACKNOWLEDGEMENT

I thank HOD, Principal, Sir MVIT and Sri Krishnadevaraya Education Trust (Sri KET), Bangalore, for their consistent support and encourage extended towards research activities.

## REFERENCES

1. Xin Zhao, MD;I,\* Xiaoxu Yang 2014 Dietary Salt Intake and Coronary Atherosclerosis in Patients With Prehypertension Cardiovascular Research Institute and Department of Cardiology, Shenyang Northern Hospital, Shenyang, China.
2. Dr Ananya Mandal, 2019 What is Oxidative stress, News Medical Life sciences.
3. Paul porier, \*Thomas D Giles 2006 Obesity and Cardiovascular Disease Pathophysiology, Evaluation, and Effect of Weight Loss, Arteriosclerosis, Thrombosis, and Vascular Biology.
4. Rosa M. Bruno, \*Lorenzo Ghiadoni 2012 Sympathetic regulation of vascular function in health and disease, Department of Internal Medicine, University of Pisa, Italy.
5. Clay F. Semenkovich 2006, Insulin resistance and atherosclerosis, Division of Endocrinology, Metabolism, and Lipid Research, Washington University School of Medicine, St. Louis, Missouri, USA.

6. Javad Sharifi-Rad \* Célia F. Rodrigues 2020, *Diet, Lifestyle and Cardiovascular Diseases: Linking Pathophysiology to Cardioprotective Effects of Natural Bioactive Compounds*, Institute for Research and Innovation in Health (i3S), University of Porto, 4200-135 Porto, Portugal.
7. Nkunda, Dominique Savio. "Effect of Purdue improved crop storage (pics) bags on the quality of stored beans (*Phaseolus vulgaris*). " *Int. J. of Agric Science and Research* 8.2 (2018): 49-54.
8. Moreno-García, Kathia L., et al. "Evaluation of the antioxidant, anti-inflammatory and antihyperglycemic activities of black bean (*Phaseolus vulgaris* L.) by-product extracts obtained by supercritical CO<sub>2</sub>." *The Journal of Supercritical Fluids* (2022): 105560.
9. Ojha, Surendra Kumar, Jane C. Benjamin, and Ajay Kumar Singh. "Mass Production of Biofertilizer (*Pseudomonas fluorescens*). " *International Journal of Agricultural Science and Research (IJASR)* 6 (2016): 415-418.
10. Dkhar, yarielyn saio & mamtaj sahana. "study on seed mycoflora of different varieties of french bean (*phaseolus vulgaris* l.). " *International Journal of Agricultural Science and Research (IJASR)* ISSN(P): 2250-0057; ISSN(E): 2321-0087 Vol. 7, Issue 4, Aug 2017, 421-428 © TJPRC Pvt. Ltd.